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ORIGINAL ARTICLE

Different Fish-Eating Habits and Cytokine Production in Chronic Urticaria with and without Sensitization against the Fish-Parasite *Anisakis simplex*

Alvaro Daschner[1], Virginia Fernández-Fígares[2], Ana Valls[1], Consolación de Frutos[1], Marta Rodero[2], Florencio M Ubeira[3] and Carmen Cuéllar[2]

ABSTRACT

Background: *Anisakis simplex* sensitization has been associated with acute, but also with chronic urticaria. The objective of this study is to characterize chronic urticaria with (CU+) and without sensitization (CU-) against the ubiquitous fish parasite *A. simplex* in a transversal and longitudinal evaluation.

Methods: 16 CU+ and 22 CU- patients were included and assessed for Urticaria activity score (UAS), fish-eating habits by standardized questionnaire and cytokine production (assessed by flow cytometric bead-based array) of peripheral blood mononuclear cells after stimulation with *A. simplex* extract or Concanavalin A (Con A). Patients were randomly put on a fish-free diet for three months and UAS, as well as cytokine production were again assessed. A difference of ≥1 in UAS was defined as improvement.

Results: There was no difference in UAS in both groups. *Anisakis* induced IL-2, IL-4 and IFN-γ production was higher in CU+. Con A induced IL-6 and IL-10 production was higher in CU+. CU+ was associated with higher total fish intake, whereas CU- was associated with oily fish intake. The correlation of UAS was positive with oily fish, but negative with total fish intake.

There was a better UAS-based prognosis in CU+ without diet. Improvement was associated with higher Con A induced IL-10/IFN-γ as well as IL-10/IL-6 ratios. Further, previous higher oily fish intake was associated with improvement.

Conclusions: Our data confirm the different clinical and immunological phenotype of CU+. Our results show a complex relationship between fish-eating habits, cytokine production and prognosis, which could have important consequences in dietary advice in patients with CU. When encountering *A. simplex* sensitization, patients should not be automatically put on a diet without fish in order to reduce contact with *A. simplex* products.

KEY WORDS

Anisakis simplex, chronic urticaria, cytokines, diet, phenotype

ABBREVIATIONS

CU, Chronic urticaria; CU+, *Anisakis simplex* sensitization associated chronic urticaria; CU-, Chronic urticaria without sensitization against *A. simplex*; PBMC, Peripheral blood mononuclear cells; Con A, Concanavalin A.

[1]Servicio de Alergia, Instituto de Investigación Sanitaria, Hospital Universitario de la Princesa, [2]Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Madrid and [3]Departamento de Microbiología y Parasitología, Facultad de Farmacia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain.
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Correspondence: Alvaro Daschner, Servicio de Alergia, Instituto de Investigación Sanitaria, Hospital Universitario de la Princesa, C/ Diego de León 62, Madrid E–28006, Spain.
Email: alvarodaschner@gmail.com
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INTRODUCTION

Chronic urticaria (CU) is a frequent and disabling illness occurring worldwide in 0.1% of the population and has repeatedly been shown to affect quality of life.[1,2] CU has been labelled autoimmune in a high percentage of cases.[3,4] But, independently of the possible autoimmune status, multifactorial genesis seems to underlie the appearance of this entity. Therefore different studies attempted at searching for different phenotypes of CU, taking into account possible infectious elicitors, mainly physical stimuli, its association with non-steroidal anti-inflammatory drug intolerance or atopy status.[5-7]

It is clear that the main shared characteristic in the heterogeneous group of CU is mast-cell activation, and the release of biogenic mediators is responsible for clinical features and the inflammatory reaction. On the other side, the knowledge on previous immunologic mechanisms leading to mast cell activation is scarce in CU, although there is now some evidence to include CU as an inflammatory disorder.[8] Some studies have highlighted different pro-inflammatory cytokines to be implicated in severity and missing regulatory features associated with this entity.[9-12]

Whereas Gastro-allergic Anisakiasis (GAA) is a well established clinical entity, where acute short-lived, IgE-mediated urticaria, angioedema or anaphylaxis accompanies acute *Anisakis simplex* infection,[13,14] a frequent phenotype of CU in our area is its association with sensitization against this fish-parasite (CU+ or *A. simplex* sensitization associated chronic urticaria).[14,15] In our area, this phenotype of CU patients constitutes up to 50% of patients attended for allergological evaluation and in a relevant subgroup of these patients sensitization against *A. simplex* was explained by previous parasitic episodes by this nematode.[15] Detection of specific IgE against *A. simplex* explains only previous parasitism in a given patient, but is not necessarily linked to a causal or temporal relationship with the onset of chronic urticaria. Thus a previous parasitic episode could be one of the predisposing factors leading to CU together with other mainly unknown eliciting factors.

The aim of characterizing phenotypes is to search for possible differentiated treatment regimes. Generally these include mainly avoidance of eliciting factors, such as in physical urticaria, drug treatment and possible dietary advice. The last has been scarce in an allergological setting due to the fact that reports on food-induced IgE-mediated elicited CU are anecdotical. On the other side, some studies have highlighted the possible role of a pseudoallergen-free diet in patients with CU.[16,17]

Sensitization against *A. simplex* is per se influenced by dietary habits, as raw fish eating has repeatedly been shown to be a risk factor for sensitization and urticaria.[18,19] The role of *A. simplex* as a hidden allergen able to elicit acute IgE-mediated clinical reactions is still under debate,[20] but *A. simplex* can be interpreted as an ubiquitous food associated antigen, and other possible immune mechanisms have not been studied so far. Further, fish is not only a possible source of biogenic amines, which could be associated with wheals,[21] but also a source of ω3 polyunsaturated fatty acids (PUFA), which could modulate an inflammatory response, as has been shown for different clinical entities, such as cardiovascular disease, rheumatoid arthritis, allergic disorders and depression.[22]

In this prospective study, our hypothesis was that CU+ and CU- differ with respect to dietary, immunological and clinical features and that consequently a temporary diet without fish would differently modulate the clinical and immunological response of patients with CU of different phenotypes.

METHODS

STUDY POPULATION

Patients were prospectively recruited for 30 months if they matched the selection criteria and gave written consent to participate. The study protocol was approved by the University Hospital La Princesa Ethical Committee.

Chronic urticaria patients were included with at least twice a week spontaneously occurring wheals for at least six weeks, independent of other conditions known to be associated with CU. Only patients with suspected food-induced urticaria and urticaria elicited exclusively by physical stimuli were excluded from recruitment. Patients who were under corticosteroids or other immuno-modulatory drugs were also excluded.

Within the group of CU, two phenotypes were differentiated: criteria to include patients in the CU+ group were a positive SPT against *A. simplex* and detectable specific IgE against *A. simplex* larval antigen. As cross-reactivity with other parasites or arthropods could theoretically explain the detection of specific IgE against *A. simplex*, a further inclusion criterion was positive serum specific IgE against *Ani s 7*, a highly specific marker of previous parasitism by this nematode, which is further the only known allergen recognised by 100% of patients.[23,24] CU- patients did not have a positive SPT nor serum specific IgE against *A. simplex*.

STUDY PROTOCOL

Severity of CU was assessed by Urticaria Activity Score (UAS). We obtained by standardized questionnaire detailed information about fish-eating habits (Table 1). As an atopic status has been associated with CU in some studies we also assessed atopy by Skin Prick test (SPT) against the most frequent aeroallergens and auto-reactivity was assessed by autologous serum skin test (ASST).

All patients were further assessed by routine labo-

Table 1 Fish-eating habits: standardized questionnaire

1. Which are the names of the fish you eat most often?
   This is a question necessary for controlling the necessary differentiation of oily and non-oily fish. †
2. How often do you eat non-oily fish in one week?
3. How often do you eat oily fish in one week?
   With these data we computed: Total fish-intake: 2 + 3.
4. How often do you eat canned fish in one week? ‡
5. How often do you eat anchovies in vinegar sauce in one month or one year?

† Most frequently referred fish consumed by patients were:
Oily fish: sardine, anchovy, swordfish, tuna, salmon, trout, red mullet, bream, seabass, salted cod. Non-oily fish: hake, blue whiting, sole, halibut.

‡ Most frequently referred canned fish consumed by patients were oily fish: tuna, sardine, mackerel.

ratory, including thyroid function, anti-thyroid antibodies and serology for Hepatitis B or C infection.

Cytokine production was measured in supernatants after stimulation of peripheral blood mononuclear cells (PBMC), stimulated with *A. simplex* antigen or Concanavalin A (Con A).

At study onset patients were randomly selected for a diet without fishery products for three months or maintaining their habitual weekly fish intake. UC+ patients, if included in the group without diet, were advised to eat previously frozen fish.

After three months all patients were again assessed for UAS and cytokine production.

Antihistamines were withdrawn five days before clinical and immunological evaluation. Otherwise, patients were asked to take the minimum number of antihistamines necessary for relief control.

URTICARIA ACTIVITY SCORE

Urticaria activity score (UAS) was assessed as previously described.[25] Shortly, severity of urticaria in CU patients was clinically assessed after withdrawing antihistamines for 5 days. The mean score of the last four days was calculated as sum of the wheal number score (between 0 and 3: 0; 0-9; 10-50; >50) and the itch severity score (between 0 and 3: no; mild; moderate; severe).

SKIN PRICK TESTS

Skin prick tests (SPT) performed were: SPT with *A. simplex* and against the most frequent aeroallergens in our area: animal dander (cat, dog), house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), pollen of *Cupressus arizonica*, *Olea europea*, *Lolium perenne*, weed mix and mould *Alternaria alternata* (ALK-Abelló, Madrid, Spain). Further, all patients were assessed for other IgE-mediated food allergies by SPT test against a set of food-agents, including egg, milk, fish, crustaceans and vegetables (Laboratories Leti, Barcelona, Spain).

SPT was performed by standard technique and was considered positive with a mean wheal diameter of 3 mm or more. Histamine at 1% concentration and saline solution 0.9% (NaCl) were positive and negative controls, respectively. Wheal diameter was measured 15 minutes after treatment.

AUTOLOGOUS SERUM SKIN TEST

ASST was performed as previously described[26] and could be performed in 23 of the studied patients. Shortly, whole blood was collected into sterile glass tubes and allowed to clot for 30 minutes. After centrifugation at 450 *g* for 10 min, an intradermal 0.05 ml injection of undiluted serum was applied at the volar forearm in parallel to the controls: 0.05 ml of normal saline as negative control and a SPT with Histamine at 1% concentration as positive control. ASST was considered positive if the mean wheal of ASST was ≥1.5 mm after 30 minutes, ensuring a positive control at 15 minutes.

CRUDE EXTRACT FROM *A. simplex*

Larvae of *A. simplex* were fragmented and sonicated and further proteins were extracted in phosphate buffer. After delipidation with n-hexane and centrifugation at 10.000 rpm at 4℃ during 30 minutes, supernatant was collected as crude extract and protein content was quantified by Bradford protein assay.[27]

SERUM SAMPLE, PBMCs AND STIMULATION ASSAY

Blood was also taken after antihistamines were withdrawn for five days. Serum was stored at -70℃ until processing and whole blood was immediately processed for stimulation assays.

The PBMC were isolated by centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient, and cell viability was assessed by means of trypan blue dye exclusion. Cells were then washed and re-suspended at 1.25×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated foetal bovine serum, 10 mM HEPES buffer, 2 mM L-glutamine, and 0.06 g/l of gentamycin. Cells cultured under stimulation with either Concanavalin A from *Canavalia ensiformis* (Jack bean) (Con A; Sigma-Aldrich) (50 μg/ml), or *A. simplex* larval crude extract (500 μg/ml), or with medium alone. Cells were also co-stimulated by means of the simultaneously addition of Con A plus antigen. Cells were incubated for 72 hours at 37℃ in a humidified incubator with 5% CO_2. Supernatants were stored at -70℃ until further processing.

LABORATORY DETERMINATIONS

In all patients we analyzed specific IgE (CAP-System, PHADIA, Uppsala, Sweden) against *Anisakis* (Cut-off point: 0.35 kU/l). Specific IgE against recombinant

Ani s 7 was also determined in order to confirm a previous parasitism.[28]

IgE AGAINST *rAni s 7*

rAni s 7 is a polypeptide with a repetitive sequence recognized by mAb UA3. When the anti-*Anisakis* IgE values for human positive sera in indirect ELISA with *rAni s 7* were compared with capture ELISA using the mAbUA3 (which recognizes *nAni s 7* allergen) all sera tested were positive by both methods.[29,30] This proves that *rAni s 7* retained the same IgE reactivity as the *nAni s 7* allergen.

Briefly, specific anti-*Anisakis* IgE antibodies were detected by indirect ELISA, with recombinant *Ani s 7* as the target. Wells of the 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were filled with 0.06 µg/well of protein. After incubation of the plates at 4℃ overnight and blocking of non-reactive sites, 100 µl of undiluted serum was added to each well and the specific IgE was detected as previously described. Optical densities (ODs) at 492 nm were calculated by subtracting the OD value produced by the same serum in the absence of antigen. The calculated cut-off values for *Ani s 7* ELISA were ODs of 0.05.[28]

CYTOKINE MEASUREMENT

Supernatants of the PBMCs stimulation assays were used for measurement of cytokine production: levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A were quantified using a multiplex assay in accordance with the instructions of the manufacturer (BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit; BD Biosciences, San Jose, CA, USA). TGF-β levels in serum were assessed by a single plex assay (BD™ Cytometric Bead Array (CBA) Human TGF-β1 Single Plex Flex Set; BD Biosciences) as indicated by the manufacturer. All samples were analyzed with BD FACSCalibur Flow cytometer™ and the results were expressed in pg/ml using the FCAP Array™ software.

EFFECT OF DIET

Clinical improvement was defined as a difference of UAS at study onset - UAS after three months (after randomization to diet/no diet) ≥1.

STATISTICS

Statistical analysis was performed using SPSS ver. 15.0 for Windows.

TRANSVERSAL EVALUATION

Prevalences were calculated for sex, atopy status and positive ASST in both studied groups and compared by Chi-square-test. Mean age, UAS, and supernatant TGF-β were calculated in all studied groups and compared by ANOVA (normal distribution of data). Anti-(IL-10, TGF-β)/pro-inflammatory cytokine (IL-6, IL-17, IFN-γ, TNF-α) production ratios were calculated and compared. Previous duration of urticaria, other cytokine levels as well as data on fish-eating habits, specific IgE and total IgE displayed no normal distribution. Here, Median and Interquartile range were calculated for and compared by Mann-Whitney. We further analyzed ratios of pro- and anti-inflammatory cytokine levels by the same methods.

Spearman correlation coefficient was used for correlation studies between cytokine production and fish intake as well as UAS.

Logistic regression was performed in order to analyze fish-eating habits and their relationship with the different urticaria phenotypes. A further regression model aimed at searching for multivariate explanation of cytokine production in both urticaria phenotypes.

Two linear regression models were applied to first analyze fish-eating habits with respect to UAS outcome and further analyze cytokine production and UAS.

EFFECT OF DIET AND PROGNOSIS

Improvement rates were compared in randomized groups (diet/no diet) in CU+ and CU- and compared by Chi-square-test. Further, improvement rates were compared in ASST+ versus ASST-, and atopic versus non-atopic patients.

Initial cytokine production was compared in patients with clinical improvement versus non-improvement. Individual cytokine changes after the clinical trial were assessed by a ratio of their post-/pre-production. Again, Median and Interquartile range were calculated for and compared by Mann-Whitney.

In patients with diet, previous fish-eating habits were compared in patients with improvement versus non-improvement.

Here, a logistic regression model was performed to analyze which factors are associated with improvement. Those variables were included initially in this model, which achieved $p < 0.1$ in bivariate analysis. Diet was also to be included as a possible explaining factor.

RESULTS

WHAT DIFFERENTIATES CU+ FROM CU-?

Epidemiology, Clinical and Routine Laboratory Data

22 CU- and 16 CU+ patients were included, 26 were female and 12 male. Mean age was higher in CU+ (54.7 ± 12.5 years old) than in CU- (39.3 ± 15.6 years old, $p = 0.02$).

13/16 patients were atopic in CU+ and 17/22 in CU-. 7/16 were sensitized against pollen in CU+ and 10/22 in CU-. 5/16 were sensitized against HDM in CU+ and 6/22 in CU- (*n.s.*).

ASST was positive in 3/12 CU+ patients and 4/11

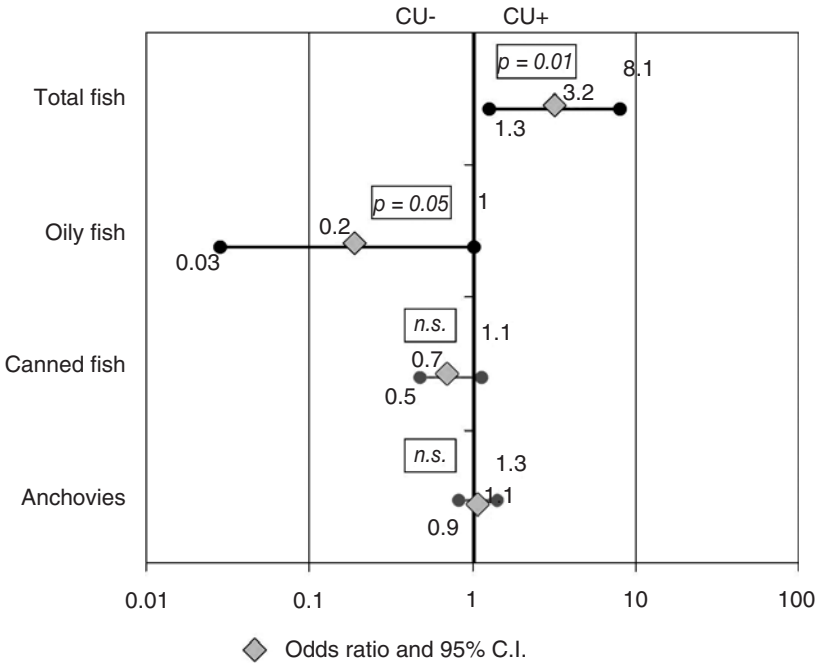

Fig. 1 Fish eating habits in different phenotypes of chronic urticaria. Results after logistic regression analysis including all possible fish-eating variables. Median weekly (total, oily fish, canned fish) and monthly (anchovies in vinegar sauce) portions of fish in chronic urticaria without (CU-) and with (CU+) previous parasitism by *Anisakis simplex*.

Table 2 Cytokine production (in pg/ml supernatant) after stimulation of PBMC with either *Anisakis simplex* extract or Concanavalin A

Cytokine induction with *A. simplex* extract								
	IL-2	IL-4	IL-6	IL-10	IL-17	TNF-α	IFN-γ	TGF-β
CU-	6.8 (0.7-51.1)	0 (0-0.1)	286 (37-576)	2 (0.3-8.1)	0 (0-8.5)	0.14 (0-1.5)	1.3 (0-7.5)	252 (162-677)
CU+	108 (37-537)	0.4 (0-1.8)	879 (43-1601)	2.4 (1.4-41)	7.2 (0-14.8)	0 (0-6.1)	10.1 (0.7-51)	390 (199-578)
P	*0.001*	*0.014*	*0.25*	*0.25*	*0.19*	*0.76*	*0.019*	*0.46*

Cytokine induction with Concanavalin A								
	IL-2	IL-4	IL-6	IL-10	IL-17	TNF-α	IFN-γ	TGF-β
CU-	18.2 (2.3-163)	1.7 (0.06-7.3)	10757 (2153-16100)	220 (103-371)	318 (59-730)	94 (26-334)	1020 (322-2902)	342 (134-741)
CU+	102 (10.6-618)	2.7 (1.0-26.5)	18152 (13016-23299)	338 (231-604)	482 (202-1283)	163 (42-522)	2758 (303-5297)	519 (308-698)
P	*0.14*	*0.23*	*0.004*	*0.03*	*0.13*	*0.26*	*0.18*	*0.24*

Median and Interquartile range in pg/ml supernatant. *P*-values are given after comparing cytokine levels in CU+ versus CU-.

in CU- (*n.s.*).

Median previous duration of urticaria was 20 Interquartile range (IQR) 8-36 months in CU+ and 6 (IQR 2.8-24) months in CU- (*p = 0.07*).

UAS was 3.6 ± 1.5 in CU+ and 4.0 ± 1.4 in CU- (*n.s.*).

Median specific IgE was 4.8 (IQR 1.5-11.5) kU/l in CU+. Median total IgE was 117 (IQR 83-414) kU/l in CU+ and 103 (43-161) kU/l in CU- (*n.s.*). Due to the inclusion criteria, all CU+ displayed IgE-antibodies against *Ani s 7*.

One CU- and one CU+ patient had a positive Hepatitis B serology, one CU- patient showed positive antibodies against Hepatitis C and one CU+ patient suf-

Table 3 Correlation studies

a) *Anisakis*		IL-2	IL-4	IL-6	IL-10	TNF-α	IFN-γ	IL-17	TGF-β
IL-2	Rho		0.466	-0.090	0.108	0.174	0.142	-0.075	0.318
	P		*0.014*	*0.654*	*0.592*	*0.394*	*0.480*	*0.715*	*0.113*
IL-4	Rho	0.466		0.237	0.565	0.486	0.561	0.434	0.183
	P	*0.014*		*0.170*	*0.000*	*0.004*	*0.000*	*0.010*	*0.307*
IL-6	Rho	-0.090	0.237		0.709	0.090	0.049	0.179	0.248
	P	*0.654*	*0.170*		*0.000*	*0.614*	*0.781*	*0.310*	*0.163*
IL-10	Rho	0.108	0.565	0.709		0.393	0.250	0.385	0.116
	P	*0.592*	*0.000*	*0.000*		*0.021*	*0.148*	*0.025*	*0.522*
TNF-α	Rho	0.174	0.486	0.090	0.393		0.400	0.347	-0.034
	P	*0.394*	*0.004*	*0.614*	*0.021*		*0.019*	*0.048*	*0.851*
IFN-γ	Rho	0.142	0.561	0.049	0.250	0.400		0.473	0.049
	P	*0.480*	*0.000*	*0.781*	*0.148*	*0.019*		*0.005*	*0.788*
IL-17	Rho	-0.075	0.434	0.179	0.385	0.347	0.473		-0.271
	P	*0.715*	*0.010*	*0.310*	*0.025*	*0.048*	*0.005*		*0.133*
TGF-β	Rho	0.318	0.183	0.248	0.116	-0.034	0.049	-0.271	
	P	*0.113*	*0.307*	*0.163*	*0.522*	*0.851*	*0.788*	*0.133*	

b) Concanavalin A		IL-2	IL-4	IL-6	IL-10	TNF-α	IFN-γ	IL-17	TGF-β
IL-2	Rho		0.746	0.246	-0.061	0.807	0.350	0.080	0.220
	P		*0.000*	*0.137*	*0.717*	*0.000*	*0.031*	*0.635*	*0.192*
IL-4	Rho	0.746		0.336	0.212	0.534	0.472	0.291	0.249
	P	*0.000*		*0.039*	*0.202*	*0.001*	*0.003*	*0.076*	*0.138*
IL-6	Rho	0.246	0.336		0.470	0.125	0.479	0.534	0.273
	P	*0.137*	*0.039*		*0.003*	*0.454*	*0.002*	*0.001*	*0.102*
IL-10	Rho	-0.061	0.212	0.470		0.052	0.538	0.709	-0.126
	P	*0.717*	*0.202*	*0.003*		*0.756*	*0.000*	*0.000*	*0.458*
TNF-α	Rho	0.807	0.534	0.125	0.052		0.412	0.132	0.253
	P	*0.000*	*0.001*	*0.454*	*0.756*		*0.010*	*0.431*	*0.130*
IFN-γ	Rho	0.350	0.472	0.479	0.538	0.412		0.528	0.144
	P	*0.031*	*0.003*	*0.002*	*0.000*	*0.010*		*0.001*	*0.396*
IL-17	Rho	0.080	0.291	0.534	0.709	0.132	0.528		0.154
	P	*0.635*	*0.076*	*0.001*	*0.000*	*0.431*	*0.001*		*0.364*
TGF-β	Rho	0.220	0.249	0.273	-0.126	0.253	0.144	0.154	
	P	*0.192*	*0.138*	*0.102*	*0.458*	*0.130*	*0.396*	*0.364*	

Spearman correlation coefficient and significancy between the production of different cytokines alter stimulation of PBMCs with either *Anisakis* (**a**) or Concanavalin A (**b**).

fered non-autoimmune hypothyroidism and was under substitution therapy.

Fish-Eating Habits

Bivariate analysis showed only total fish intake to be significantly higher in CU+ (2.5, IQR 1.6-2.5 versus 1.25, IQR 1-2.5 portions per week; $p = 0.04$).

Figure 1 highlights results on fish-eating habits after regression analysis, which shows CU+ to be highly associated with total fish intake (B = +3.2; CI 1.3-8.1; $p = 0.01$), whereas CU- was associated with oily fish (B = 0.2; CI 0.03-1.0; $p = 0.05$). Canned fish and anchovies in vinegar sauce are not significantly associated with both urticaria phenotypes.

Cytokine Production

In a general manner, all studied *Anisakis*-stimulated cytokines were produced in higher quantities in CU+ than in CU-, but highly significant differences could be stated for IL-2, IL-4 and IFN-γ (Table 2).

But also Con A stimulated PBMC were higher in CU+ and reached significant differences for IL-6 and IL-10 (Table 2).

Correlations between cytokines can be seen on Table 3. Overall, cytokine production was frequently positively correlated with each other.

Regression analysis did not yield valuable results of cytokine production explaining CU+ or CU-. No

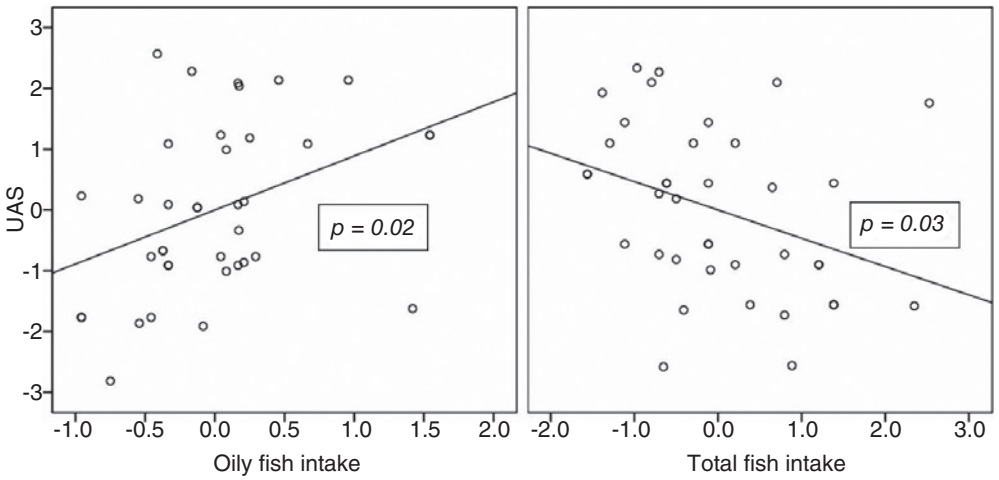

Fig. 2 Urticaria activity score (UAS) and fish-eating habits. Graphic of partial regression: those variables are shown, which achieved $p \leq 0.05$ in regression model. All variables including data on fish-eating habits were included in this model in order to search for explaining UAS outcome.

significant results could be stated for the ratios of pro- and anti-inflammatory cytokine production.

Relationship between Data-Groups

In patients with CU+, total fish intake was correlated with Con A induced IL-6 and IL-17 production (Rho > 0.5; $p < 0.05$).

In CU-, total fish intake was associated with *A. simplex* induced IL-2 (Rho 0.46; $p < 0.05$), but negatively correlated *A. simplex* induced TNF-α (Rho = -0.53; $p = 0.02$). Oily fish intake was also associated with *A. simplex* induced IL-2 production (Rho 0.56; $p = 0.01$). Anchovies in vinegar sauce were negatively associated with TNF-α and IFN-γ (Rho < -0.53, $p < 0.02$). Canned fish was negatively correlated with IL-4 (Rho 0.44; $p = 0.05$) production.

Regression analysis including cytokine results showed only Con A induced IL-6 to be associated with CU+, but with a negligible odds ratio (Odds ratio 1.0003 CI 1.000-1.-0006; $p = 0.05$).

UAS was not associated with the production of specific cytokines, but in CU+ UAS was positively correlated with higher *A. simplex* induced IL10/IL17 (Rho 0.63; $p = 0.02$) as well as IL10/IFN-γ (Rho 0.51; $p = 0.05$) ratios, and in CU- positively with IL10/TNF-α (Rho 0.48; $p = 0.04$) but negatively with TGF-β/TNF-α ratio (Rho -0.51; $p = 0.03$).

UAS was also associated with fish-eating habits. Regression analysis demonstrates UAS to be positively associated with oily fish intake (B = 0.89; $p = 0.02$), but negatively with total fish intake (B = -0.47; $p = 0.03$) (Fig. 2).

WHICH FACTORS AFFECT THE PROGNOSIS OF CU?

Bivariate Analysis

We could not find a positive effect of diet on improvement of CU+, there was rather a tendency to a better outcome if patients were not under diet ($p = 0.07$). No such effect was detected in CU-. There was no different outcome with respect to positive ASST.

When analysing only CU+, improvement was associated with atopy (mainly when HDM sensitized, $p = 0.03$). No such effect was found in CU-.

Initial high Con A induced IL-10/IFN-γ ($p = 0.009$) as well as IL-10/IL-6 ($p = 0.07$) ratios were associated with improvement.

When comparing individual post- and pre-trial cytokine production, in CU-, improvement was associated with higher *Anisakis* induced TNF-α ($p = 0.04$), IFN-γ ($p = 0.01$) as well as Con A induced IFN-γ production ($p = 0.04$), whereas Con A induced TGF-β production was lower ($p = 0.01$). In CU+, improvement was associated with a higher *Anisakis* induced TGF-β production ($p = 0.04$).

Multivariate Analysis

Regression analysis showed that both diet, but also previous higher total fish intake were associated with a worse prognosis, whereas previous higher oily fish intake was associated with a better prognosis (Fig. 3a).

Again, when analysing only patients who were put on diet, these improved when they have previously been eating higher amounts of oily fish and lower amounts of total fish (Fig. 3b).

DISCUSSION

Our results confirm that CU+ and CU- have, besides the presence of specific IgE, some different clinical and immunological characteristics. CU+ patients were older than CU-. This fact is in concordance with previous studies, which showed *A. simplex* sensitization and parasitism to be associated with raw fish eat-

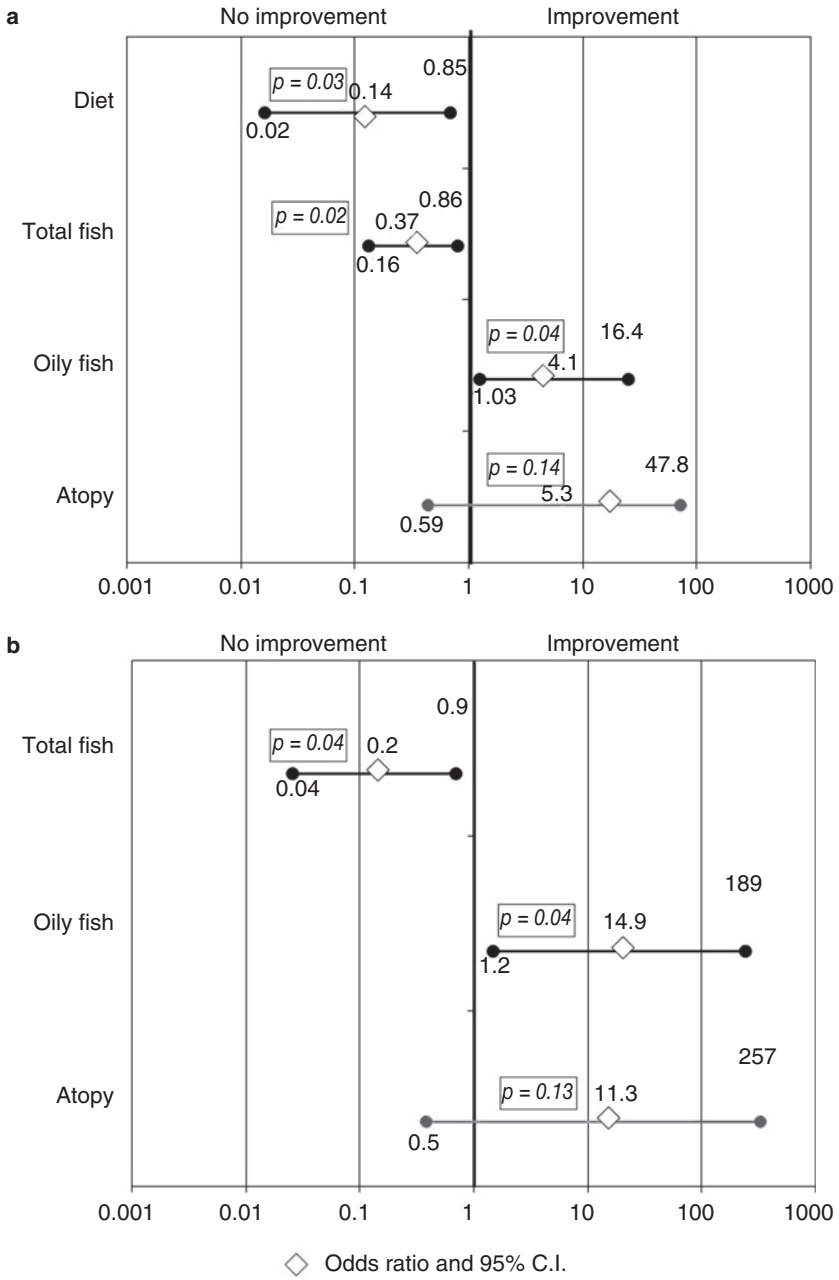

Fig. 3 Explaining variables for clinical improvement of chronic urticaria. Improvement is defined as a minimum decline of Urticaria activity score of 1 after the trial. Those variables were included initially in this model, which achieved $p < 0.1$ in bivariate analysis (fish-eating habits, diet, atopy status). **a**) Here diet was also included as a possible explaining factor and achieves significance for a worse prognosis. **b**) Analysis of only those patients who were put under diet without fishery products.

ing habits (in our region anchovies in vinegar sauce) in older populations. We cannot otherwise rule out that urticaria in its acute or chronic phenotype appears, like in other immune conditions, after a certain threshold of accumulated contact with *A. simplex* antigen.[31] As we included in our CU+ only those patients who also displayed IgE against *Ani s 7* and thus suffered previous parasitism, the different age-patterns confirms that previous parasitism is really at least one causal factor leading to the appearance of CU+. The recognition of this allergen is sufficient to prove a previous parasitic episode and known pan-allergens,

such as tropomyosins or paramyosins from other sources, which have their equivalent as *Ani s 2* and *Ani s 3*, have not been proven to elicit clinically relevant cross-reactions after parasitism by *A. simplex* and thus were not in the scope of this study.[20]

Raw fish-eating habits have been associated both with sensitization against *A. simplex* and GAA as well as CU+.[18,31] Our study did not find a significant difference in the frequency of raw fish eating (anchovies in vinegar sauce), but two reasons could be discussed: first, perhaps the low number of studied patients. Second, we know that social alarm about *A. simplex* infections and legal requirements concerning control of parasites in fish to be consumed raw in the last decade have highly impacted the population with respect to fish-preparation and this fact could impact on our results.

A previous study showed that raw fish eating was the main risk factor for IgE production against *A. simplex* and less the quantity of total fish intake.[18] Our own results show that total fish intake is associated with CU+, compared to CU-. This is due to the higher probability of contact with live larvae due to the second risk factor, undercooked fish, which leads to detectable specific IgE.[13,32]

More interestingly, the amount of oily fish-intake is independently associated with CU-. Further, UAS was both dependent on the amount and type of fish intake. One possibility explaining UAS to be positively correlated with oily fish intake is the already known presence of biogenic amines mainly in oily fish, which are partly responsible for UC- and severity.[33] On the other side oily fish intake has previously been shown to be associated with a decreased production of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-α,[11,12] a fact that would not fit with these data in the transversal evaluation. But independently of fish-eating habits, cytokine production shows the same seemingly counterintuitive direction, with UAS correlating with higher Con A induced IL-10/IL-17 as well as IL10/IFN-γ and *Anisakis* induced IL-10/TNF-α ratios. Similarly, IL-10 production by mitogen has been verified in previous studies involving patients with CU.[10,11] We could not verify IL-6 production to be associated with the severity of urticaria, as proposed in another study.[12] But the negative association of UAS and the *Anisakis* induced TGF-β/IL-6 ratio in CU- renders emphasis to these cytokines in a plausible direction. Here IL-6 could be interpreted as a marker of systemic inflammation as well as a marker of disease activity in CU.[12] On the other side of the anti-pro-inflammatory axis, TGF-β could be implicated in dampening the inflammatory reaction, lowering UAS. We should otherwise not forget that we are not assessing a control population and that our results could be specific when analysing CU.

How can we interpret these data? As we have seen, pro- as well as anti-inflammatory cytokine production is highly correlated with each other, when stimulated with the same agent. Thus, in CU it seems that the significant factor leading to activity of urticaria is missing in our analysis and only epiphenomena are detected. Biological processes are marked by a steady tendency towards a regulation of disequilibrium as in inflammation. It is thus possible, that we find associations, such as in this case, which seem counterintuitive, but fit well when cytokines with antagonistic properties are found on the same axis, such as association with higher versus lower UAS. Expectedly, the production of several cytokines by *A. simplex* is higher in CU+ patients reaching statistical significance for IL-2, IL-4 and IFN-γ. Interestingly, not only Th2- associated IL-4, but also Th1-associated IFN-γ is higher here. GAA in humans, as well as acute parasitism in a murine model is associated with a simultaneous Th1 and Th2 type response, which parallels our findings.[34-36] But also several Con A stimulated cytokines were higher in CU+, with significant results for IL-6 and IL-10. This is interesting, because it reflects an overall higher reactivity of cytokine secreting cells in patients with previous parasitism, not only for specific but also for unspecific stimuli. Thus, in patients in whom previously an active parasitism had stimulated their cells in vivo with specific antigens, these cells are specifically stimulated in vitro with the antigens from the parasite resulting in a Th1/Th2 response typically for helminth infections. These responses were previously demonstrated by us and other authors in human and animal models.[34-36] On the other side, total fish intake was higher in CU+ patients, but oily fish intake was lower. We could hypothesize that intake of ω3 PUFA could be lower in CU+ patients and consequently their anti-inflammatory effects were reduced.

TNF-α, IL-17 and TGF-β values were those cytokines to demonstrate most similar production in both CU phenotypes after specific or unspecific stimulation. TNF-α up-regulation has been shown in skin of different types of urticaria.[37] Increased TNF-α as well as IL-10 production was also detected in patients with chronic idiopathic urticaria.[11] Further TNF-α is significantly increased in sera form chronic idiopathic urticaria patients and is one of the implicated factors contributing to the skin lesions seen in CU, with higher IL-17 secretion when ASST was positive.[10] TGF-β, which has positive as well as negative effects on mast-cell function and survival, has been proposed as a pro- as well as anti-inflammatory cytokine.[7] A previous study showed higher circulating serum TGF-β levels to be associated with the chronic urticaria phenotype in *Anisakis* sensitized subjects.[38] As we were not able to show difference of this cytokine between CU+ and CU-, it is possible that circulating TGF-β levels do not reflect the local production.

Thus, it can be argued that in CU, previous parasitism has no measurable effect on those cytokines pre-

viously known to be produced in higher quantities, whereas cytokines of the Th1/Th2 axis are differentially stimulated by *Anisakis* in CU+, and anti-/pro-inflammatory IL-10 and IL-6 are unspecifically stimulated in CU-.

The clinical relevance of CU+ as a differential entity was further assessed by measuring the clinical and immunological effect of a diet without fishery products, which also parallels absence of contact with *A. simplex* products.

Improvement was unexpectedly not associated with diet in CU+. Rather, the opposite effect was stated: patients who continued to eat fish had a better UAS outcome. Our results show a complex relationship between fish-eating habits, cytokine production and prognosis, which extends to the whole group of CU patients. Improvement is not only related to the continuation of fish consumption, but also to previous fish-eating habits with higher oily fish consumption associated with improvement, but an opposite effect of total fish intake. Higher initial anti-/pro-inflammatory cytokine ratios showed further a significant association with improvement. In CU+ TGF-β production changed to higher values in those who improved. However, CU- patients showed changes towards higher pro-inflammatory cytokine and lower TGF-β production in those who improved.

These results highlight again the fact that cytokine production and their possible anti- or pro-inflammatory properties and the influence on UAS depend on the urticaria phenotype studied and possibly on other multiple factors. Together, possible factors affecting the immunological and clinical phenotype of CU as well as the prognosis with or without diet will not only depend on contact with *A. simplex* products, but more importantly on other factors associated with fish-eating habits, which again affect the ratio of pro- and anti-inflammatory cytokines.

Taken together, by phenotyping CU with respect to previous parasitism, we can propose an interesting model, where a complex interaction of *A. simplex* associated balance between Th1/Th2, but also a pro- and anti-inflammatory balance (IL-10/TGF-β- IL17/IL-6) modulated also by fish intake is associated not only with the phenotype of CU, but also with the prognosis and the outcome when put under a fish free diet.

Dietary intervention should therefore not only take into account the possible missing eliciting factors, but also the possible missing protective factors. When encountering *A. simplex* sensitization, patients should not be automatically put on a diet without fish in order to reduce contact with *A. simplex* products. Future studies could now complete this analysis using biomarkers of fish-consumption in order to strengthen our findings based on a questionnaire.

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